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MORPHOLOGY AND FUNCTION OF REGENERATING MONOAMINERGIC AND CHOLINERGIC NEURONS

A STUDY IN THE ADULT RAT USING CEREBRAL IRIS TRANSPLANTS AS TARGETS

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Niels-Aage Svendgaard

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AKADEMISK AVHANDLING

som med vederbörligt tillstånd av Medicinska Fakulteten i Lund för avläggande av medicine doktorsexamen kommer att offentligen försvaras å Histologiska avdelningens föreläsningssal (nya byggnaden), Institutionen för anatomi och histologi, Lund, tisdagen den 3 juni 1975 kl. 9.15 (prick)

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To E. Varnaa Jensen

The present summary is based on the following papers:

I Regenerative properties of central noradrenaline, dopamine and indolamine neurons in the adult rat brain using transplants of irides as target, Ergeb. Anat. Entwicklungsgesch., 51/4 (1975) 1-77. (Together with A. Björklund and U. Stenevi).

II Regeneration of central cholinergic neurons in the adult rat brain. To be published. (Together with A. Björklund and U. Stenevi).

III Reestablishment of functional connections by regenerating central adrenergic and cholinergic axons, Nature, 253 (1975) 446-447. (Together with A. Björklund, B. Johansson and U. Stenevi).

IV Axonal degeneration associated with a defect blood-brain barrier in cerebral implants, Nature. In press. (Together with A. Björklund, JE Hardebo and U. Stenevi).

The papers will be referred to by their Roman numerals.

Abbreviations used: ACh, acetylcholine; AChE, acetylcholinesterase; CA, catecholamine(s); CNS, central nervous system; DA, dopamine; DTB, dorsal tegmental CA bundle; 5,6-DHT, 5,6-dihydroxytryptamine; IA, indolamine(s); MFB, medial forebrain bundle; NA, noradrenaline; PBZ, phenoxybenzamine; 6-OH-DA, 6-hydroxydopamine.

"But the functional specialization of the brain imposed upon the neurons two great lacunae; proliferative inability and irreversibility of intraprotoplasmic differentiation. It is for this reason that, once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated."

CAJAL (1928)

INTRODUCTION

Our knowledge of the structural and functional properties of the central nervous system (CNS) goes back to the 19th century and has expanded rapidly during the last two decades. Golgi's silver chromate method provided the possibility of visualizing nervous elements and their relationships to one another in a far better way than previously available. During the eighties and nineties many investigators, among them Cajal and His, using the Golgi technique, created the modern morphological concepts of the structure of the central nervous system. Mainly on the basis of their investigations, Waldeyer (1891) formulated the now generally accepted neuron doctrine: The nervous system is built up by units, neurons which are independent of each other from anatomical as well as genetical points of view. A neuron is a nerve cell with all its processes which are, peripherally and centrally, connected with the processes of other cells by means of contact only.

In the study of the formation of nervous connections terms such as chemotropism, chemotaxis, galvanotaxis, and neurotropism were commonly used by

Cajal (1928 a and b) and others at the beginning of this century to explain selectivity in the formation of nervous connections. During a period, aspecially in the 1930´s and 40´s, alternative theories to explain these concepts of selectivity were dominant. The "theory of homologous response" and the "resonance theory" of Weiss (1941) were developed on the basis of denervation and reinnervation experiments in peripheral nerves in lower vertebrates. In this theory, which has been well discussed in the review by Weiss (1941), the nerve fibres are supposed to grow out and form connections randomly and non-selectively with the effector organs. Functional specificity in the system was inferred by Weiss from his resonance and modulation theories. These theories on neuro--effector contacts were attacked by Sperry in 1939. Sperry not only took up the chemotropism ideas of Cajal, but went far beyond this, suggesting an "individual identification tag" that might be cytochemical in nature, and that was responsible for a selectivity not confined to regions in the CNS, but could operate at the level of the individual nerve cells. A series of impressive experiments on the central and peripheral nervous system of lower vertebrates (Piatt 1942, 1952, 1957; Sperry 1945, 1963; Mark 1965; Marotte and Mark 1969; Sperry and Arora 1965) provided support for the theory of Sperry.

The question of structural reorganization or plasticity in the CNS of the adult mammal has always been a controversial subject. Recently, theories of a continuing structural modification of synaptic organization as an explanation of memory function has been suggested (see e.g. Rosenzweig et al. 1972). Although structural modification is today recognized as a basic mechanism for functional plasticity in the brain, there is, however, no clearcut experimental evidence proving this point. Plasticity as a response to injury is a much easier model for experimental study. A growth response to damage of CNS tissue may occur in two basically different ways. First, in the form of regenerative sprouting that occurs after transection of an axon. The distal part of the fibre degenerates and the proximal stump sends out axonal sprouts. Second, in the form of collateral sprouting that occurs in a partially denervated area, where the remaining axons form collateral sprouts which reinnervate the denervated sites. The possibility that mature differentiated neurons in the CNS might

show regenerative responses has until recently been regarded with pessimism (for review see Cajal 1928; Windle 1955; and Clemente 1964). Cajal described what he called an abortive regeneration, i.e. a regenerative sprouting started from lesioned axons, but was followed by atrophy and resorption within 4-5 weeks. One approach to the problem of CNS regeneration has been to study the regrowth of central axons into transplants of peripheral tissue placed in the CNS. This technique has given very different results. An ample ingrowth of axons into CNS implants was reported by Tello (1911), Shirai (1935) and Horvat (1966, 1967, 1969). Contradictory results were obtained by Le Gros Clark (1942, 1943) and by Bernard and Carpenter (1959) who denied every form of significant regrowth of central neurons. Le Gros Clark explained the appearance of nerves in the grafts of Shirai and Tello as being partly due to dislocation of preexisting fibres by edema and cellular infiltration, or partly to a traction effect imposed on the host tissue by the transplant.

Common to all these investigations is, above all, the use of non-specific silver staining techniques. Cranial sympathectomy was, moreover, never performed to prevent ingrowth of sympathetic fibres from the pial vessels into the graft. In addition, the not very successful results of Le Gros Clark might be explained by the rather limited survival of his transplants. Due to these limitations, the earlier studies using transplantation techniques do not provide a conclusive answer as to whether or not a regeneration occurs in the CNS.

Thanks to the recent rapid development of neuro-morphological techniques (especially histochemical and ultrastructural techniques), the study of neuronal growth has met with a renaissance. The introduction in 1962 by Falck and Hillarp of a sensitive and specific fluorescence method, which allowed a localization of monoamine transmitter substance in the cell bodies and axon terminals in the CNS (Falck et al. 1962; Falck 1962; for reviews see Corrodi and Jonsson 1967; Björklund et al. 1972), was a great stimulus to investigate the growth properties of the monoaminergic neuron systems. The Falck-Hillarp method allowed a detailed mapping of the adrenergic neurons, both in the peripheral and central nervous system (Dahlström and Fuxe 1965; Ungerstedt 1971; Lindvall and Björklund 1974). In 1949 Koelle and Friedenwald elaborated a method for staining of AChE. A variety of biochemical, histochemical

and physiological studies suggest that AChE-positive fibre systems are in all probability cholinergic (for discussion see paper II). The Koelle method therefore offers a useful technique for morphological studies of cholinergic fibre systems in the CNS (Shute and Lewis 1963, 1967; Lewis and Shute 1967).

These histochemical techniques have provided better tools for studies of growth phenomena in the CNS due to their high specificity and sensitivity, allowing studies on neuron systems identified on the basis of their transmitters or enzyme content. In addition, the introduction of the neurotoxic compounds 6-OH-DA and 5,6-DHT has been very useful in growth studies as they make possible selective lesioning of monoamine-containing axons in the CNS. In recent years these new neuromorphological methods have provided good evidence that a substantial collateral sprouting can take place in at least certain neuron systems within the CNS (Goodman and Horel 1967; Raisman 1969; Raisman and Field 1973; Moore et al. 1971; Pickel et al. 1974). Studies on central monoaminergic neurons in the adult rat have revealed a high capacity for axonal sprouting after both mechanical (Katzman et al. 1971; Björklund and Stenevi 1971; Björklund et al. 1971) and chemical axotomy (Nygren et al. 1971, 1973; Björklund et al. 1973; Nobin et al. 1973). Furthermore, in embryonic brain stem tissue transplanted to the anterior eye chamber, monoamine neurons have been shown to grow actively over a denervated iris (Olson and Seiger 1972, 1973).

The present series of experiments which are summarized below are mainly based on the use of transplantations to the CNS as models for the study of central regeneration. This technique of transplanting a peripheral tissue to the upper brain stem in order to study the regenerative capacity of the central monoamine neurons, was introduced by Björklund and Stenevi 1971. The technique as it was used at that time had several drawbacks. It did not give any information about the distribution and patterning of the regenerating monoamine fibres within the graft. Nor did it disclose the mode of growth of, or interactions between the different monoamine systems. Finally, no information was obtained as to whether the regenerated central axons could reform functional synaptic contacts in the transplants.

By using aseptic conditions during the initial surgery, it proved possible

to extirpate at the time of sacrifice the iris graft from the transplantation bed in the brain, and to unfold the iris on a microscope slide as a whole mount preparation for subsequent microscopy. By transplanting a portal vein instead of an iris to the brain this technique also made it possible to obtain a specimen for functional evaluation of regenerated synaptic contacts in a field stimulation chamber. The present study is a detailed investigation of the regenerative growth of lesioned NA, DA, IA and AChE-positive neurons into irides transplanted to various sites in the brain. The conditions for survival of cerebral implants as well as development of blood-brain barrier properties in them were also investigated.

MATERIAL AND METHODS

Adult albino Sprague-Dawly rats were used. Autologous transplantations of peripheral tissue to the CNS were used in all the works (paper I-IV) with a few exceptions. Neonatally sympathectomized irides (paper I), portal veins (paper III) and embryonal CNS tissue were transplanted homologously.

Operative procedures

The rats were anaesthetized with a barbiturate (Brietal, Lilly, 40 mg/kg, i.p.) or ether. Great care was taken in order to achieve aseptic conditions during surgery.

Transplantations (papers I-IV). Iris grafts were most commonly used (papers I, II, and IV). The iris was dissected out and transferred to steril Ringer solution immediately before transplantation. In paper III 7-8 mm long sections of portal veins were opened longitudinally, and the whole or half of the circumference was used. Embryonic CA neuron containing brain tissue in about 1 mm^2 big pieces was obtained from embryos of a crown rump length of 15-30 mm (paper IV). Transplantation to the brain. A stereotaxic instrument was used. A craniotomy was made in the perietal bone and the dura incised and opened. Four different positions (paper I) were chosen stereotaxically. Two different positions were used in paper II, and only one position in papers III and IV. The lowering of the graft itself was made by free hand with a thin glas rod. Transplantations to the eye. This technique was essentially that of Olson and Malmfors (1970). The tissue to be transplanted (iris, fragment of sympathetic ganglion,

or small block of embryonic brain tissue) was sucked up into a thin glass tube filled with Ringer solution and connected to a reservoir. The graft was transferred to the anterior chamber of the recipient eye by injecting the tissue via a corneal incision (paper I, II, and IV). Lesions of locus coeruleus (paper I). A unilateral lesion of locus coeruleus was made stereotaxically with a radiofrequency lesion generator (Radionics RFG 4), using a microme wire electrode. Lesions of ascending NA fibre pathways in the brain stem (paper I), were made stereotaxically by bilateral intracerebral injections of 6-OH-DA. Denervations. Cervical sympathectomy (papers I-IV) was performed by bilateral removal of the superior cervical ganglia. Ciliarectomy (paper I) was made by extirpation of the ciliary ganglion on one side. Chemical neonatal sympathectomy (paper I) was carried out by consecutive daily intraperitoneal injections of 6-OH-DA during a 5-day period.

Perfusion technique

Suspensions of fluorescent plastic particles (methyl metacrylate spheres, labelled with fluorescein) (paper I), Indian ink, and tryphan blue (paper IV) were used as perfusion mediums. The chest was opened under general barbiturate anaesthesia. Via an incision in the left ventricle, a plastic catheter was led into the ascending aorta and kept tightly in position by a ligature. At the start of the perfusion the right ventricle was opened and the descending aorta clamped. Immediately after the perfusion the animals were decapitated.

Dissection procedures

The animals were killed under a light ether anaesthesia (papers I-III) or under a general barbiturate anaesthesia (paper IV) at times varying between one day and one year after the operation. The transplants were either prepared as whole mounts (papers I, II and IV), as in situ preparations (papers I-IV), or the portal vein grafts (paper III) were freeze-dried after field stimulation. Finally, the embryonic tissue transplants to the anterior eye chamber (paper IV) were freeze-dried together with the host iris. In preparing the whole mounts, the brains or the eyes were rapidly removed, and the iris grafts dissected out and unfolded on a microscope slide with a pair of fine watch-makers tweezers under a stereo-microscope. The irides were dried in a desiccator over phosphorous pentoxide for at least two hours. In the in situ investigations (papers

I, III, and IV), the brain region containing the transplant — and in some cases (paper I) also the electrothermic lesion or the 6-OH-DA injection sites — was dissected out in one block. The tissue piece was immediately frozen in a liquid propane-propylene mixture at the temperature of liquid nitrogen, and freeze--dried.

Histochemical procedures

All the whole mounts in paper I and IV and a part of freeze-dried specimens in paper I-IV were prepared for fluorescence microscopy according to the method of Falck and Hillarp (Falck 1962; Falck et al. 1962; for technical details see Björklund et al. 1972). The whole mount preparations were processed at 80°C for one hour with gaseous formaldehyde of a relative humidity of 70-75 %. The specimens were mounted in Entellan containing xylene (paper I) or in liquid paraffin (paper IV). The freeze-dried material was reacted with formaldehyde at 50 % relative humidity. Sections were cut at 5-10 µ and mounted in Entellan or liquid paraffin on glass slides for fluorescence microscopy.

The whole mount preparations in paper II, and part of the freeze-dried material in paper II and III that was cut in a cryostat at 20 µ thick sections, were stained for AChE according to the Holmstedt (1957) modification of the Koelle copper thiocholine method for AChE. Incubation time was 4-20 hours, and Mipafox was used as inhibitor of non-specific cholinesterases. In addition one group of whole mounts (paper II) was prepared for combined visualization of CA- and AChE-containing fibres. After drying, the specimens were treated with formaldehyde vapour at room temperature for three hours. The unmounted specimens were photographed in the fluorescence microscope, and, after that, incubated for staining of AChE in the presence of Mipafox, as above.

Microspectrofluorometry

For characterization of the formaldehyde-induced fluorophores, (primarily in order to distinguish between DA- and NA-containing fibre systems in the transplants, a modified Leitz microspectrofluorometer was used (see Björklund et al. 1968, 1972).

Field stimulation experiments

The portal vein transplant (paper III) was removed from the brain and

mounted in an organ bath containing buffered, oxygenized Krebs solution at 38°C for combined isometric recordings and electrical field stimulation. The spontaneous activity of the transplant as well as the effect of NA, ACh or electrical field stimulation on the isometric tension (with or without an α--adrenergic blocker or atropin) was investigated. Field stimulation was carried out with square wave impulses of 15 V amplitude between the electrodes (5 mm apart), 1.0 msec. duration, and 6 or 10 Hz (Axelsson et al. 1967; Johansson et al. 1970).

RESULTS AND COMMENTS

1. Vascularization and survival of ocular and cerebral implants

The start and further development of revascularization of transplants to the anterior eye chamber and to the brain were evaluated after perfusion with a suspension of fluorescent plastic particles and a subsequent examination in the fluorescence microscope. In both types of transplantation, the recirculation started at day 1 and was fully developed in the eye transplants at day 5. In the CNS implants the progressive revascularization was well developed at day 5, but not completed until after 2-3 weeks. There were only small — if any — changes of the vascular density of the cerebral implants between three weeks and two months after operation.

The rate of survival was high. Less than 10 % of the CNS implants were totally necrotic, and only about every fourth transplant showed minor necrotic areas. The best survival rate was found in grafts receiving their vascular supply both from the brain parenchyma and from pial vessels at the base of the brain and from the choroidal fissure. Other factors such as sterility and absence of haematoma were of major importance for the survival of the CNS implants.

In the cerebral implants a pronounced size reduction occurred fully comparable to that observed by Olson and Malmfors (1970) and Malmfors et al. (1971) in transplants to the anterior eye chamber.

Investigations of revascularization of ocular and CNS implants using e.g. dyes or isotopes have not been reported before in the literature. The recirculation in the ocular grafts was observed to start earlier than has been previously reported (Olson and Malmfors 1970; Malmfors et al. 1971). Le

Gros Clark (1942) observed microscopically vessels bridging between the brain tissue and a CNS implant. Medawar (1948) made similar observations and had good survival results. It can be concluded that the brain is an excellent transplantation site, not much inferior to the anterior eye chamber.

In a series of experiments, the revascularization and the reinnervation processes were compared. It was found that the vessels grew in before the nerves, and extended in the iris ahead of the sprouting fibres. The vessels and the nerve grew out over the iris in a pattern that principally resembled each other, but they seemed to grow independently of each other. The regenerated nerve fibres were never seen to expand into a non-vascularized area.

2. The ingrowth, morphology, and patterning of central NA fibres in irides transplanted to the caudal diencephalon. (Paper I and IV).

In order to demonstrate the mode of reinnervation by central NA fibres of an iris transplant to the caudal diencephalon, the iris was extirpated and prepared as a whole mount preparation for fluorescence microscopy 5 to 120 days after operation. A few specimens were treated as in situ preparations at 6 and 12 months after surgery. To avoid interference with sympathetic neurons the animals underwent cranial sympathectomy. The main source of the reinnervating central NA fibres was the dorsal tegmental bundle (DTB), while a minor contribution originated from the medial forebrain bundle (MFB). The latter pathway also mediated DA and IA fibres to the transplant (see Ungerstedt 1971 and Lindvall and Björklund 1974).

The first sprouting NA fibres were observed in the iris five days after transplantation. At day 9 loose irregular fibre bundles, morphologically resembling the fibres of origin in the DTB, expanded over about 1/3 of the iris. Independent of the site of ingrowth (ciliary body, dilator or sphincter) the fibre bundles had a tendency to expand circularly, especially in the ciliary body and the sphincter, where they circled for longer distances than the fibres of the normal adrenergic innervation. The growth rate between the fifth and ninth day was roughly estimated at 0.5-0.8 mm/day of the DTB fibres. Between day 9 and 15 the branching bundles reached across the whole

iris, and a beginning development of fibre plexuses was observed. At four weeks after transplantation, the ingrowing fibres, initially resembling DTB bundles, had developed terminal plexuses that covered practically the whole iris, and they had successively become denser and more regular and mature in morphology mimicking the normal sympathetic nerve supply. At longer survival times (2 and 12 months after operation) the regenerated fibres were well preserved in some specimens, but in the major part a progressing degeneration of the nerve plexus was observed.

These investigations are to some extent comparable to those reported by Olson and Seiger (1972, 1973) who transplanted embryonic monoaminergic neurons to the anterior chamber of a sympathectomized eye. Also in this experimental situation the central NA, DA and IA neurons are seen to ramify out over the iris. However, these authors found no signs of degeneration in the long-term groups. The results demonstrated attracting, organizing and modulating influences of the iris on the regrowing central NA sprouts, and it seemed as if this was due both to an attraction of the growing sprouts across the necrosis and to a stimulation of growth and arborization of the new fibres within the transplant.

3. Reinnervation of CNS implants by DA and IA fibres in the absence of NA axons.

It is well known from previous studies (Katzman et al. 1971; Björklund and Stenevi 1971; Björklund et al. 1971) that central DA and IA neurons possess a high capacity for sprouting. In order to offer the transected DA axons a selective contact with the iris, the graft was placed either in the internal capsule, where it transected the dopaminergic nigrostriatal pathway, or in the caudal diencephalon in contact with the damaged MFB in animals that had the ascending CA fibre systems eliminated prior to the transplantation through a bilateral intracerebral injection of 6-OH-DA in the caudal mesencephalon. Two points of time, 16-19 days and 1 month were chosen for investigation. To exclude interference by NA fibres the preparations were controlled by microspectrofluorometric analyses. At the site of ingrowth delicate fine varicose fluorescent fibres at 16-19 days penetrated the entire muscle layer

and expanded to cover, at the most, only 25-30 % of the iris. The branching and subsequent patterning of the DA fibres did not clearly mimick the pattern of the normal autonomic ground plexus of the iris, suggesting that the DA axons were poorly related to the Schwann cell sheaths. After one month no definite further outgrowth or morphological maturation of the DA fibres could be observed.

Through an electrothermic lesion of the homolateral locus coeruleus prior to grafting, or through weekly intravenous injections of 6-OH-DA (see Material and Methods), an iris transplanted to a position proximal to the mesencephalic raphe nuclei was brought in contact with the transected ascending IA fibres in the absence of regenerating NA axons. The grafts were examined 16-19 days and one month after the transplantation. Because of technical difficulties met with when demonstrating the IA fibres in whole mounts, part of the specimens were treated as in situ preparations. At 16-19 days the IA fibres from an area of massive ingrowth, where they penetrated the entire muscle layer, expanded in loosely arranged bundles to reach about 2-3 mm across the iris. At one month they reached, in some specimens, to cover practically the entire transplant. In restricted areas of the dilator the fibres formed organotypic plexuses, although neither as extensive nor as complete as those formed by the central NA sprouts.

The central NA, and to some extent also the central IA fibres, demonstrated an ability to form organotypic terminal patterns; the central NA and IA were in some cases seen to do so running close together. In contrast, the patterns formed by the ramifying DA axons were notably defective and showed no clear resemblance to the autonomic ground plexus of the normal iris. The results agree with the findings of Olson and Seiger (1972, 1973). They transplanted embryonic CNS tissue to the anterior chamber of the eye. Judging from their photographs there is a marked resemblance in the mode of regrowth of the central monoaminergic fibres in the two different experimental situations.

4. Interaction between sympathetic, central NA, DA and IA neurons competing for the same target. (Paper I).

If the sympathetic nerve supply to the brain is left intact, peripheral NA axons are observed to grow into a transplant in the caudal diencephalon from the pial vessels and from the plexus of sympathetic fibres in the habenular region. These regenerating peripheral fibres are morphologically distinguishable from the central ones in the iris graft between 5 and 15 days after operation and can be recognized in the fluorescence microscope (see above). At five days, the ingrowth of sympathetic fibres was markedly more advanced than that of the central axons. At day 9 the presumed peripheral fibres formed fairly well-developed plexuses occupying about one quarter of the iris. In one group of animals sympathectomized 12 days after transplantation and killed at day 15, a defective or markedly reduced extension of the central DTB fibres in the graft was observed compared to the ingrowth of central NA fibres in a control group sympathectomized before transplantation (see A in Fig. 1). If the sympathetic fibres are removed, the central NA fibres will expand over the entire iris, but the DA sprouts will remain restricted to the region close to their site of ingrowth, and the IA sprouts will enter the transplant only to a very limited extent (B in Fig. 1). At this position of the transplant, the removal of the source of the regenerating central NA sprouts (see above), allows the regrowing DA fibres to expand out over part of the transplant, whereas the IA fibres will still remain very restricted (C in Fig. 1). A similar pattern of the DA fibres is obtained in a sympathectomized animal by transplanting an iris to the internal capsule (D in Fig. 1). In a graft transplanted caudal to the mesencephalic DA cell groups in a sympathectomized rat with a lesion of the ascending CA axons, IA sprouts will expand over the entire graft (E in Fig. 1). These observations seem to reveal differences between the various monoaminergic neurons as regards their affinity for or their response to the tissue normally supplied with sympathetic nerves. In the peripheral sympathetic nervous system, Olson and Malmfors (1970) have shown that different types of postganglionic adrenergic neurons seem able to readily replace each other.

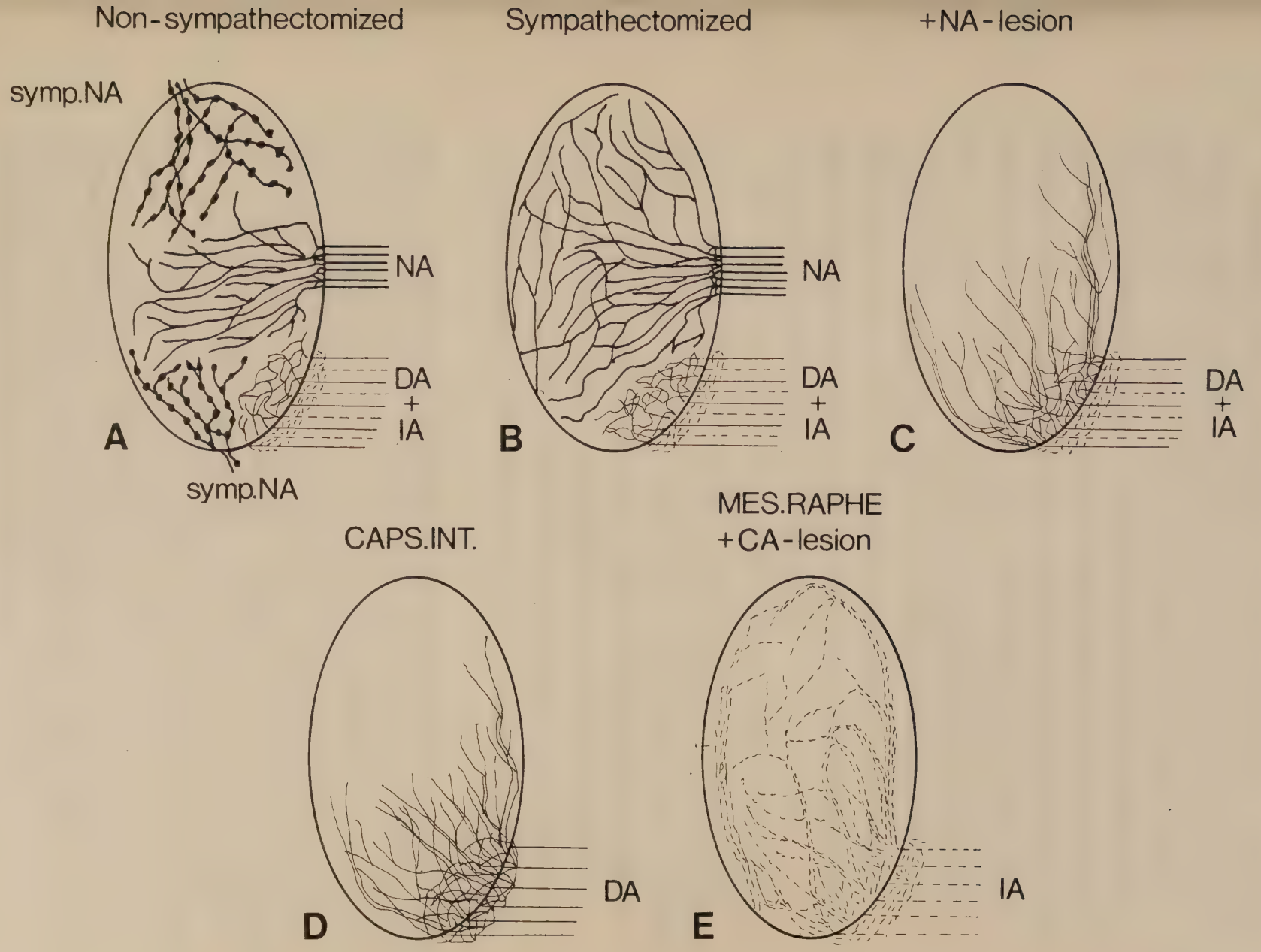
Non-sympathectomized
Sympathectomized
+NA-lesion
symp.NA
NA
DA
+
IA
A
symp.NA
B
C
CAPS.INT.
MES.RAPHE
+CA-lesion
DA
D
IA
E

Fig 1A-E

5. Growth of central NA fibres into chronically denervated irides. (Paper I)

Tello (1911), in his studies on the sprouting lesioned central axons into cortical implants, suggested that the release of an active principle from the neural elements during the acute phases of degeneration, might actively stimulate the axonal outgrowth. Such a possible role seemed of particular interest after the demonstration of the effect of NGF on the sprouting of central NA neurons into irides transplanted to the CNS (Björklund and Stenevi 1972; for review see Björklund et al. 1974). Chamley et al. (1973) and Chamley and Dowel (1975) have demonstrated in in vitro experiments that tissues normally densely innervated exert a stronger attraction and growth stimulating effect than not sympathetically innervated tissue on neurons specifically susceptible to the action of NGF. They ascribed this property to the release of NGF from the cultured tissue.

In one type of experiments, new-born rats were treated by five consecutive daily injections of 6-OH-DA. In this way irides were obtained that had never had any sympathetic innervation. In another experiment, the eyes of adult rats were sympathectomized or sympathectomized and parasympathectomized 1-3 monts before transplantation to the caudal diencephalon. The specimens were prepared for fluorescence microscopy 18-20 days and one month after operation. No difference in the reinnervation was observed

Fig. 1. Diagrams representing schematically principal features of the ingrowth and extent of sprouting of the different types of monoaminergic neurons — sympathetic NA (~), central NA (~), DA (~) and IA (~) — in irides transplanted under the various experimental conditions used in this work. A: Non-sympathectomized host animal. Sympathetic and central NA fibres compete with each other in the reinnervation of the transplant, whereas the DA and IA sprouts remain restricted to the region close to their site of ingrowth. B: Sympathectomized host animal. Central NA fibres expand over the entire iris, whereas the DA and IA sprouts remain restricted as in Fig. A. C: Sympathectomized host animal with a lesion of the ascending NA fibres. DA sprouts expand over large parts of the transplant, whereas IA sprouts still remain restricted. D: Transplant to the nigrostriatal DA pathway in a sympathectomized host animal. DA sprouts behave principally as in Fig. C. E: Transplant implanted caudal to the mesencephalic DA cell groups in a sympathectomized host animal with a lesion of the ascending CA fibres. In the absence of growing CA fibres, IA sprouts expand over the entire graft.

between the neonatally and adult denervated animals. Nor was any difference observed in the time-course of ingrowth and patterning of the larger fibre bundles and fibre strands in these transplants compared to the control groups. But the terminal network was notably dense and irregular in contrast to the more ordered plexuses in transplants of acutely denervated irides.

The attracting influence of the iris on the regrowth of lesioned central NA axons are, thus, not confined to the acute phases of denervation or to the degeneration of neural elements in the target tissue. The defective development of the terminal plexus might well be related to the atrophy of the smooth muscle in the grafts that could be observed at the time of transplantation.

6. Regeneration of central cholinergic neurons into cerebral implants. (Papers II and III)

The regeneration of lesioned central AChE-positive axons in the adult rat was studied in irides transplanted to two different brain sites: in the caudal diencephalon and hippocampus, and in the hippocampal fimbria. The grafts were prepared partly as whole mounts, partly as in situ preparations and treated for microscopy by AChE-staining according to Holmstedt (1957). For comparison between the reinnervation process by central and peripheral cholinergic axons, irides were also transplanted to the anterior eye chamber. The microscopical evaluation was made at several points of time between 12 days and 2 months. From the in situ preparations, it could be determined that the main contribution site of sprouts of the AChE-positive, presumably cholinergic axon, appeared from the transected septo-hippocampal pathways. Growth of newly-formed AChE-positive fibres into the transplant was also observed from lesioned axons in the anterior thalamus, and to a minor extent from the dorsal and ventral tegmental AChE-positive pathways and the habenulo-interpreduncular tract.

In the whole mount preparations from both transplantation sites, it was observed that, during the first 2-4 weeks after transplantation, the sprouting central fibres grew out over large areas of the iris. The new fibres branched profusely into a terminal plexus that covered maximally about half of the

iris surface, and in some areas the patterning of the regenerated central fibres mimicked closely that of the normal autonomic cholinergic innervation of the iris. At 60 days no further progression of the reinnervation could be noted. Parts of the transplants were markedly involuted, and in such areas the regenerated AChE-positive fibre networks looked defective.

In one series of experiments, the AChE-staining was combined with fluorescence histochemical visualization of regenerated adrenergic fibres in the same specimens, according to Ehinger and Falck (1966). In many areas there was a striking congruity in the distributional patterns of the regenerated central cholinergic and adrenergic fibres in the transplant. This indicates that — as in the normal iris — the sprouting cholinergic and adrenergic axons regenerate together along the denervated Schwann cell sheaths.

From the comparison between the central reinnervation process and the process of reinnervation of the iris by peripheral cholinergic axons after transplantation to the anterior eye chamber, it can be concluded that the regenerative capacity of central cholinergic neurons is not much inferior to that of their peripheral counterparts.

7. Functional evaluation of central adrenergic and cholinergic fibres regenerated into a portal vein graft

The portal vein is known to have an adrenergic innervation, while a cholinergic nerve supply has not been demonstrated in the normal rat portal vein, although muscarinic cholinergic receptors are present (Johansson *et al.* 1970; Häggendal *et al.* 1972). A portal vein (see Material and Methods) was transplanted to the caudal diencephalon. The animals were sacrificed after 25-30 days. In one group of specimens microscopical investigation with the Falck-Hillarp fluorescence method, and with the Holmstedt (1957) modification of the Koelle method for AChE, revealed a substantial reinnervation of most of the transplants by NA-containing and AChE-positive nerves, respectively. In another group, the grafts were mounted in an organ bath for combined isometric recordings and electrical field stimulation and after completion of the *in vitro* experiments, the specimens were prepared for fluorescence microscopy (see Material and Methods). It is well established

(Paterson 1965; Holman et al. 1968; Johansson et al. 1970) that transmural stimulation of the type used in this study selectively excites the nerves in the tissue. A majority of the specimens showed a spontaneous activity comparable to that of the normal portal vein. In the grafts displaying spontaneous activity, field stimulation evoked contractions of the same magnitude as those elicited by NA and ACh added to the bath. Phenoxybenzamine (PBZ) blocked the field stimulation responses totally in some, but only partially in other transplants. In these PBZ-resistant specimens, atropine abolished the contractions completely. Similar results were obtained also when the blockers were added in the reverse order. The response of the portal vein transplant to electric field stimulation was in all probability due to NA released from adrenergic nerves and ACh released from cholinergic nerves in the transplants as the effect was blocked by their respective blockers. Microscopical analyses of the in vitro stimulated transplants showed a good correlation between the PBZ-sensitive contractile responses to field stimulation and the presence of NA-containing fibres in the transplant.

The ability of regenerating central neurons to establish synaptic contacts in vivo has not been described previously. Interestingly, in a recent study Hoffer et al. (1974) investigated the maturation process of cerebellar cortex tissue transplanted to the anterior chamber of the eye. It was found by electrophysiological and histological examinations that the synapses in the cerebellar grafts developed and matured in a homotypical way without the normal contact with the rest of the brain. The combined histochemical and physiological data in this study provide strong evidence for functional neuro-muscular connections being formed by the regenerated central adrenergic and cholinergic axonal terminal networks in the transplanted portal veins. An important implication of this study is that — when given the same growth conditions as in the peripheral nervous system — at least some neuron systems in the mammalian CNS will have the ability to restitute a normal neurotransmission in a denervated tissue.

8. Blood brain barrier defects and their possible role for damage of regenerated central axons in cerebral implants

The capillaries in the CNS contain barrier mechanisms preventing substances from passing into the brain parenchyma from the blood. The capillaries of the rat constitute an enzymatic trapping mechanism for certain monoamine precursors (Bertler et al. 1964, 1966). Thus, when administered systemically, L-dopa (L-3,4-dihydroxyphenylalanine) was found to be taken up into the wall of the brain capillaries in which it was decarboxylated to DA. DA could be domonstrated using the fluorescence method of Falck and Hillarp.

In order to demonstrate whether or not these barrier properties exist in the rich vascularized cerebral implants of peripheral tissue, irides were transplanted autologously to the caudal diencephalon and the barrier towards L-dopa was tested at several intervals between 3 and 60 days. For comparison, the barrier problem was investigated in transplants of CNS tissue to the anterior eye chamber. The rats were treated with nialamide and L-dopa before sacrifice. The iris transplants that receive their vascular supply from the brain parenchyma and the pial vessels did never exhibit any fluorescent capillaries. In the embryonic CNS tissue to the anterior eye chamber — being revascularized from the peripheral vessels in the host iris — the capillaries obtained a normal strong fluorescence. These observations suggest that the barrier properties of the new circulation are determined not by its origin but by the properties of the revascularized tissue.

The lack of a normal blood-brain barrier for amines in the grafted irides was further substantiated by intravenous injections of 6-OH-DA that resulted in a total destruction of the regenerated NA axons in the iris up to the site of ingrowth from the brain tissue.

Striking similarities exist between the 6-OH-DA-induced axonal damage and the axonal retraction and resorption occurring in the long-term specimens (2-4 months) described in section 3. In the pituitary gland, a region that normally lacks blood-brain barriers, the central innervation shows abundant ultrastructural and fluorescence histochemical signs of acute damage. This

phenomenon has been suggested to represent a continuous degeneration--restitution cycle (Baumgarten et al. 1972 and Dellman and Rodriguez 1970).

These observations suggest that the integrity of the blood-brain barrier is necessary for the protection of cerebral tissue from structural damage. If such were the case, the nature of the factor or factors in the plasma — under normal or pathological conditions — responsible for the axonal damage, is open to speculation. Berry and Riches (1974) and Ferringa et al. (1973), for example, have proposed that abortive regeneration in the CNS is due to an inhibition of axonal regrowth by immunological mechanisms mobilized through an injury.

CONCLUSION

The present histochemical and physiological investigation has demonstrated a reinnervation of iris or portal vein, transplanted to the CNS, by monoaminergic and cholinergic neurons. The central adrenergic and cholinergic fibres create a terminal pattern hardly distinguishable from that of their peripheral counterparts, while the IA and especially the DA fibres seem to be defective in replacing adrenergic neurons in the reinnervation of the graft. Field stimulation experiments in vitro demonstrated the formation of functional synaptic contacts between the regenerated central adrenergic and cholinergic fibres and the smooth muscle in the graft. A degeneration or resorption of the regenerated axons in the long-term transplants is associated with a defective blood-brain barrier in the vessels of the implants.

The present demonstration of significant regeneration in central neurons raises the question of what clinical implications such phenomena may have. The study demonstrates a strong experimental evidence that axonal sprouting in the CNS can create functional terminal connections. Axonal and collateral sprouting are most probably two of the basic mechanisms underlying functional plasticity in the CNS. Functional recovery after brain lesions or surgical lesions such as spinothalamic tractotomy might very well be related to sprouting from remaining intact axons or from axons in a parallel system.

The tenacious idea — e.g. Cajal's idea of proliferative inability of central neurons as quoted in the introduction — of a difference in regenerative capacity between peripheral and central neurons in mammals does not seem to be of general validity. The present study shows that the regenerative capacity of at least some types of central neurons is quite comparable to that of peripheral nerve cells. The study indicates the great importance of an adequate interaction between a nerve cell and the target. A situation that does not seem to exist very often after injury of the CNS. In future work it is important therefore to search for means to stimulate axonal regrowth and obtain adequate conditions for reconnections of regnerating neurons and their appropriate target structures in the CNS after traumatic lesions. In all likelihood the mechanisms regulating the regrowth of central adrenergic and cholinergic axons does not differ decisively from those regulating other types of central neurons. To the extent it is so, the monoaminergic and cholinergic neurons should be the ideal models for the study of these problems.

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